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IDENTIFICATION OF 'RICKETTSIA RICKETTSII' IN FORMALIN-FIXED, PA--ETC(U)
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Identification of Rickettsia rickettsii in Formalin-Fixed, Paraffin-Processed Tissues by Immunofluorescence

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Running head: IDENTIFICATION OF R. RICKETTSII

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Abstract

With slight modification of a trypsin digestion technique, Rickettsia rickettsii were demonstrated specifically by immunofluorescence in formalin-fixed, paraffin-embedded tissue sections from a human, rhesus monkey and guinea pig with Rocky Mountain spotted fever, and infected membranes from a chicken embryo. Tissues were cut at 4 μm and, using gelatin as a tissue adhesive, were hydrated in a routine manner. Sections were then digested in refrigerated 0.1% trypsin for 16 hours, washed and stained specifically for R. rickettsii by direct or indirect immunofluorescence. Rickettsial organisms were localized in affected vessels of the mammalian species and within the yolk sac epithelium of the chick embryo. Specificity was assured by adsorbing antibody conjugates with R. rickettsii organisms. Trypsin digestion probably decreased tissue proteins which interfered with immunochemical attachment of antibody to the rickettsiae. The technique is valuable in that a diagnosis of Rocky Mountain spotted fever can be confirmed from formalin-fixed tissues processed in a routine manner.

(Key words: Rocky Mountain spotted fever; immunofluorescence; rapid identification)

RICKETTSIA RICKETTSII is typically difficult to demonstrate histochemically in tissue sections because of (1) its small size, (2) poor staining contrast between the organisms and tissues, and (3) the affinity of the stains for normal tissue structures such as mast cell granules which, because of their size and morphology, can be mistaken for rickettsiae[12]. These problems are encountered with a variety of special stains including Giemsa[4], Wright[11], Gimenez[4], and Pinkerton[11]. Because of these difficulties, and the lack of specific identification of R. rickettsii with the foregoing methods, immunofluorescence (FA) has been relied upon to demonstrate rickettsial organisms in pathogenetic studies of Rocky Mountain spotted fever (RMSF) [5-7, 9] and in human tissues submitted for diagnostic confirmation of the disease[12]. This technique is preferred to routine histochemical procedures because of the ease of locating rickettsiae within tissue and the immunochemical specificity of the antibody conjugates. The primary drawback of FA to date, however, has been that it is necessary to examine sections from unfixed frozen tissues.

Repeated attempts in our laboratories to demonstrate rickettsial organisms by FA in tissues fixed with 10% neutral formalin, were uniformly unsuccessful. With slight modification of a trypsin digestion procedure for fixed tissues, we were able to identify R. rickettsii by FA in formalin-fixed, paraffin-embedded tissue sections from an experimentally infected rhesus monkey, guinea pig, and chick embryo yolk sac. In addition, R. rickettsii were demonstrated in tissues from a fatal human case of RMSF.

Materials and Methods

Specimens

Paraffin blocks containing formalin-fixed tissues from a guinea pig, rhesus monkey, and chick embryo previously inoculated with R. rickettsii (Sheila Smith strain) were retrieved from storage in Pathology Division. The guinea pig was given 10^7 R. rickettsii intraperitoneally and was killed 3 days later. The monkey had been inoculated subcutaneously with 10^4 R. rickettsii and was killed 9 days later. The 5-day-old chick embryo was inoculated in the yolk sac with 10^5 R. rickettsii and killed 3 days later. The inocula were prepared as previously described[5,6]. The paraffin blocks of tissue from each accession had been in storage approximately 4, 3, and 1 years, respectively. Testis and epididymis from the monkey and guinea pig, and yolk sac from the chick embryo were examined. Frozen tissues and paraffin blocks of formalin-fixed lung, heart, and testis from the human case of RMSF were referred for immunofluorescent confirmation. Diagnosis of RMSF was established by the contributors of this case by culture of R. rickettsii from fresh tissues, electron microscopic observation of rickettsial organisms within tissues, and characteristic clinical signs and lesions. Acetone-fixed smears of R. rickettsii grown in cell culture were also stained with the various antisera to determine effectiveness of the adsorption procedures.

Antisera

Antiserum against R. rickettsii was obtained from a previously infected monkey. A portion of this serum and goat anti-monkey gamma globulin were each conjugated with fluorescein as previously described[5,7].

Tissue Preparation and Staining

The procedures of Huang et al.,[1] were employed with the following modifications. Rather than using LePage Bond Fast resin glue (LePage's Ltd, Montreal, Canada) we found that gelatin, the tissue mordant routinely used in our laboratory, resisted trypsin digestion and did not autofluoresce. Approximately 0.2g of gelatin (Bacto-Gelatin, Difco) was sprinkled over the surface of a 2-liter 37 C water bath and tissue sections were floated from the water bath onto precleaned glass slides. Excess water was drained and the slides were placed on a 60 C warmer for 1 hour. The tissue sections were deparaffinized, hydrated, and immersed in a 0.1% solution of trypsin 1:250 (Difco) and 0.1% $CaCl_2$ in distilled water with the pH adjusted to 7.8 with 0.1 N NaOH. Tissues were incubated overnight (16 hours) in the trypsin solution at 4 C, rinsed thoroughly in distilled water and placed in phosphate-buffered saline for 30 minutes prior to staining. Direct and indirect staining was carried out as previously described.[5,7]

The direct procedure was controlled by staining normal tissue with fluoresceinated monkey anti-RMSF globulin and infected tissue with either fluoresceinated rabbit anti-Q fever, or the fluoresceinated monkey anti-RMSF globulin which had been adsorbed with R rickettsii. Controls for the indirect technique consisted of (1) staining normal tissue with hyperimmune monkey anti-RMSF globulin and fluoresceinated goat anti-monkey globulin, (2) direct staining of infected tissue with only fluoresceinated goat anti-monkey globulin, (3) reacting the sections with RMSF-negative monkey serum and applying the fluoresceinated goat anti-monkey globulin, and (4) reacting the section with monkey anti-RMSF (adsorbed with R. rickettsii) and fluoresceinated goat anti-monkey globulin.

Fluoresceinated and untagged anti-RMSF globulin were exhaustively adsorbed by reacting samples of each with R. rickettsii organisms. The antigen used for adsorption of immune globulin was grown in static chick embryo cell culture and killed with 0.1% formalin. Pellets of approximately 1.5×10^8 rickettsiae were added to 1 ml aloquots of immune serum and continually mixed in the dark for 1 hour at 37°. A portion of each serum without the addition of R. rickettsii was treated identically. Each sample was spun twice at 5,000 x g; the supernatant was removed and readsorbed as described above. The supernatant and paired unadsorbed sera were used to stain tissue sections.

Results

Microscopic examination of testis and cremaster muscle from the guinea pig and monkey revealed segmental vascular necrosis and inflammation. These lesions were characterized by perivascular accumulation of mononuclear cells, fibrinoid degeneration of the media of larger vessels, degenerative and proliferative endothelial changes, and thrombosis of some of the more severely affected vessels. In the human, microscopic alterations of vessels were minimal and consisted principally of congestion of pulmonary capillaries accompanied by exudation, and moderate congestion of vessels in the testis. Tissues from the chick embryo and embryonic membranes were essentially normal.

Using trypsin digestion, R. rickettsii were demonstrated in the endothelium of affected vessels with both direct and indirect immunofluorescent procedures on formalin-fixed, paraffin-embedded tissues of the three mammalian species. In addition, rickettsiae were encountered in the endothelium of unaltered vessels of the heart and testis of the man, and in the yolk sac epithelium of the chick embryo (Fig. 1). The organisms appeared as small pleomorphic rods or coccobacilli. Intensity of staining was brighter in the indirect as compared to the direct method; staining intensity was slightly reduced in formalin-fixed, paraffin-embedded tissues compared with fresh human tissues and smears of R. rickettsii grown in cell culture. The morphology of formalin-fixed, paraffin-embedded tissues in this immunofluorescent procedure was markedly improved over that of frozen tissues so that identification of various cells and tissues harboring rickettsiae was readily apparent. Normal tissues without rickettsiae did not stain; conjugates not specific for R. rickettsii

and adsorbed anti-RMSF globulin failed to demonstrate rickettsiae both on smears and within frozen and fixed tissues containing the organisms.

Discussion

A common method for immunofluorescent examination of tissues is to section frozen tissues, fix in acetone or alcohol, and stain with fluoresceinated conjugates.[8] We have found this procedure to be inconvenient during experimental studies of RMSF, and in retrospective diagnoses, fresh tissues are often unavailable. Formalin-fixed material on the other hand is usually available.

Except for some antigens such as those in certain bacteria[10], mycoplasma[3], fungi[2], and immunoglobulins[1], tissue fixation in formalin frequently precludes subsequent demonstration of the antigens by FA. Repeated earlier attempts to identify spotted fever rickettsiae in nontrypsinized formalin-fixed tissues were unsuccessful. Trypsin treatment of the formalin-fixed, paraffin-embedded tissues permitted specific immunochemical attachment of antibody to R. rickettsii. The exact mechanism is not known[1] but it may result from removal of non-specific protein which interfere with the staining of rickettsiae by labeled antibodies[2]. With this treatment, staining intensity of rickettsiae was only slightly reduced in the formalin-fixed, paraffin-embedded tissues compared with nontrypsinized frozen tissues from the human case of RMSF stained in an identical manner. However, as indicated previously, tissue morphology was better in the formalin-fixed tissue so that cellular morphology and localization of the rickettsiae were readily apparent[1].

The attachment of the antibody to rickettsial organisms was indicative of specificity because (1) the organisms were morphologically typical of R. rickettsii, (2) organisms were associated with vascular lesions similar to published accounts of RMSF in the mammals, (3) organisms were found by FA in sections of frozen tissue from the human case of RMSF, and (4) antiserum adsorbed with R. rickettsii failed to stain the rickettsiae in replicate tissue sections from the human case, and in smears of R. rickettsii grown in tissue culture.

The procedure of trypsin digestion as described by Huang et al.[1] was modified by replacing the tissue adhesive with gelatin. Since this is a mordant commonly used by many pathology laboratories, the preliminary steps do not deviate from routine histotechnology procedure. Thus, routine unstained sections submitted to the pathologist can be utilized for FA. The advantages of this technique in a diagnostic situation especially in retrospective tissue examination are readily apparent in that (1) a diagnosis of RMSF can be made on autopsy or biopsy tissue specimens processed in a routine manner, (2) specific identification of R. rickettsii can be made in infected tissues, and (3) risk of potential infection by sectioning frozen tissues is eliminated by fixation in formalin. Preliminary results indicate that the technique is equally applicable to other rickettsiae of the spotted fever group (Rickettsia conorii and Rickettsia sibirica) as well.

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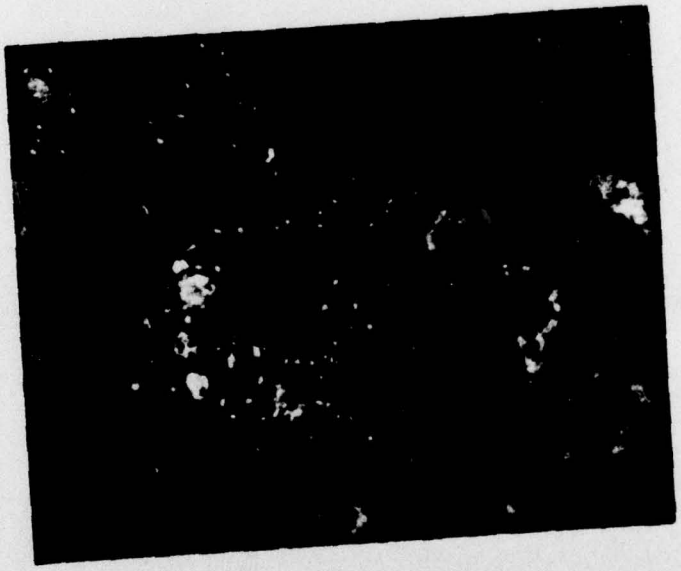
Figure Legend

Fig 1. R. rickettsii in chick embryo yolk sac epithelium.

Direct immunofluorescence X400.



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4